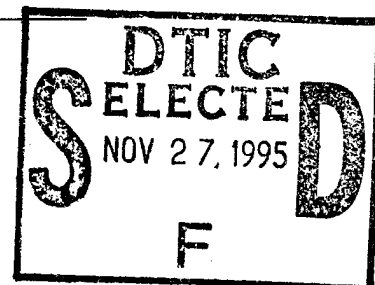


AD \_\_\_\_\_



GRANT NO: DAMD17-94-J-4257

TITLE: TGFa-myc Interactions in Mammary Tumorigenesis

PRINCIPAL INVESTIGATOR(S): Robert B. Dickson, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University  
Washington, DC 20057

REPORT DATE: September 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19951124 057

DTIC QUALITY INSPECTED 1

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1995	3. REPORT TYPE AND DATES COVERED Annual 1 Sep 94 - 31 Aug 95	
4. TITLE AND SUBTITLE TGFA-myc Interactions in Mammary Tumorigenesis			5. FUNDING NUMBERS DAMD17-94-J-4257	
6. AUTHOR(S) Robert B. Dickson, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20057			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT  Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)  This project sets out to establish <i>in vivo</i> , the basis for a dramatic cooperation between the <i>c-myc</i> oncogene and transforming growth factor $\alpha$ (TGF $\alpha$ ). These two genes are commonly amplified or overexpressed respectively in human breast cancer. We will use a novel bitransgenic model to overexpress the two genes in the mouse mammary gland. In addition, we will use mammary epithelial transplant procedure to establish whether this interaction can occur when TGF $\alpha$ and Myc interact in a paracrine fashion. Finally, the expression of several human breast cancer associated genes will be studied in tumors which arise. In the first year of study, we successfully established the timecourse, sex dependence, and organ preference for TGF $\alpha$ - Myc interactions in our system. In addition, we characterized the expression of both transgenes and the epidermal growth factor receptor in mammary and salivary tumors which arose.				
14. SUBJECT TERMS		Growth Factor TGF $\alpha$	Myc Oncogene	EGF receptor Transgenic
		15. NUMBER OF PAGES 25		
		16. PRICE CODE		
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABSTRACT Unlimited

## GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to *stay within the lines* to meet *optical scanning requirements*.

**Block 1. Agency Use Only (Leave blank).**

**Block 2. Report Date.** Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

**Block 3. Type of Report and Dates Covered.** State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

**Block 4. Title and Subtitle.** A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

**Block 5. Funding Numbers.** To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C - Contract	PR - Project
G - Grant	TA - Task
PE - Program Element	WU - Work Unit Accession No.

**Block 6. Author(s).** Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

**Block 7. Performing Organization Name(s) and Address(es).** Self-explanatory.

**Block 8. Performing Organization Report Number.** Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

**Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es).** Self-explanatory.

**Block 10. Sponsoring/Monitoring Agency Report Number.** (If known)

**Block 11. Supplementary Notes.** Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

**Block 12a. Distribution/Availability Statement.**

Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

**DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."

**DOE** - See authorities.

**NASA** - See Handbook NHB 2200.2.

**NTIS** - Leave blank.

**Block 12b. Distribution Code.**

**DOD** - Leave blank.

**DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.

**NASA** - Leave blank.

**NTIS** - Leave blank.

**Block 13. Abstract.** Include a brief (*Maximum 200 words*) factual summary of the most significant information contained in the report.

**Block 14. Subject Terms.** Keywords or phrases identifying major subjects in the report.

**Block 15. Number of Pages.** Enter the total number of pages.

**Block 16. Price Code.** Enter appropriate price code (*NTIS only*).

**Blocks 17. - 19. Security Classifications.** Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

**Block 20. Limitation of Abstract.** This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

LBD Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

LBD Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

LBD In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

LBD In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

LBD In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Robert B. White 9/26/95  
PI - Signature Date

## TABLE OF CONTENTS

Item	Page
Introduction	5
Body	6 - 9
Conclusions	10 - 11
References	12
Appendix	13

Accession For		1
NTIS	CRA&I	<input checked="" type="checkbox"/>
DTIC	TAB	<input type="checkbox"/>
Unannounced		<input type="checkbox"/>
Justification		
By		
Distribution /		
Availability Codes		
Dist	Avail and/or Special	
A-1		

## INTRODUCTION

These studies were carried out to evaluate the potential tumorigenic interaction of *c-myc* (a nuclear oncogene) and transforming growth factor  $\alpha$  (TGF $\alpha$ , a growth factor). Previous work in human breast cancer has identified *c-myc* as a gene which is frequently amplified and over expressed. In addition, the growth factor TGF $\alpha$  is commonly overexpressed in breast cancer, along with other members of its family, including amphiregulin (1). While neither TGF $\alpha$  nor *c-myc* overexpression has been shown alone to be a strong prognostic indicator in the disease, our previous work had established that the two gene products strongly synergised *in vitro* to promote transformed growth of human mammary epithelial cells in soft agar colony assays (1,2). The purpose of the present work was to utilize transgenic mouse methodology to establish the malignancy - promoting interactions of TGF $\alpha$  and Myc in breast tumorigenesis. The tumors will be studied for expression of the transgenes, markers of differentiation, and of several other malignancy- associated genes. Finally, we will use xenograft transplantation of different genotype mammary cells combined in variable proportions and implanted in different genotype recipient mammary fatpads *in vivo* to asses TGF $\alpha$  - Myc paracrine interactions.

## BODY

The goals of the research in the first year were two- fold. First, we wished to establish the time course of hyperplasia and tumor development in bitransgenic Myc/TGF $\alpha$  mice. Second, we wished to establish the range of paracrine models whereby TGF $\alpha$  is produced by one mammary cell type (stromal or epithelial) and allowed to interact with Myc - expressing mammary epithelial cells to promote their malignant function.

We used bitransgenic mating methodology (3) to co-overexpress the growth factor TGF $\alpha$  and nuclear oncogene *myc* in mouse mammary glands. Tables 1 and 2 summarize the striking results. A strong synergy was observed between the two gene products, leading to 100% incidence of mammary tumors with a mean onset time of  $66 \pm 12$  days in both male and females. The tumors were classified as adenocarcinomas types A and B [see also histopathology in figure 1 in Ammundadottir et al (3), in the Appendix].

*Table 1* Number of offspring in each of four genotype groups

Mean mammary gland tumor onset times and tumor frequency in each group is presented.

Genotype	No. of animals (%) <sup>a</sup>	Mean tumor onset time (days)	Tumor incidence
TGF $\alpha$ /c-myc	23 (15)	66 $\pm$ 12	100% <sup>b</sup>
TGF $\alpha$	24 (15)	NA	0%
c-myc	39 (25)	298 $\pm$ 55	50%
Wild type	71 (45)	NA	0%

<sup>a</sup> Total offspring number is 157.

<sup>b</sup> One hundred % equals 20/20 animals; 3 animals died at very young ages. NA, not available (tumors were not observed for more than 1 year).

*Table 2* Summary of histopathological findings in mammary glands of transgenic mice at 3 and 7 months of age

Genotype	3 mos.	7 mos.
TGF $\alpha$ /c-myc	Multiple adenocarcinomas (types A and B) in females and males <sup>a</sup>	NA
TGF $\alpha$	Normal	Cystic ducts
c-myc	Normal	Atypical hyperplasia to adenocarcinoma
Wild type	Normal	Cystic ducts

<sup>a</sup> Histopathology from both virgin females and males. All other diagnoses are from virgin females only. NA, not available (all animals of this genotype are deceased at this time point).



We next established the time course of tumor onset in males and females. We designed these experiments with the knowledge that upon necropsy (usually about 3 months of age), all bitransgenic, tumor bearing animals exhibited non-palpable malignancies in all other mammary glands. In addition, no normal tissue was observed in any mammary gland of any animal at this time (3). We carried out whole mount preparations to study the morphology of control and bitransgenic glands weekly from approximately 3-6 weeks of age. This is the time period during which the epithelial rudiment elongates and penetrates the mouse mammary fatpads. Surprisingly, at just 24 days of age, we could reproducibly observe tumorous appearing nodules, disrupted morphology, and enlarged ducts, [this can be observed in figure 2 of the Appendix, Amundadottir et al (3)]. Excision and reimplantation of these early tumors subcutaneously in nude mice yielded rapidly growing, invasive cancer (3).

We examined tumors and found, as expected, that they expressed high levels of TGF $\alpha$  and myc mRNA's by northern blot [see Figure 3 in Appendix, Amundadottir et al (3)] and by *in situ* hybridization [see Figure 4 in Appendix, Amundadottir et al (3)]. The epidermal growth factor receptor (EGFR) was also expressed in the tumors [see Figure 6a, b, in Appendix, by Amundadottir et al (3)].

A second goal of our first year of study was to explore possible paracrine actions of TGF $\alpha$  to drive Myc- dependent tumorigenesis, since our bitransgenic model cannot readily discriminate autocrine paracrine, and endocrine modes of growth factor action. We initially excised mammary epithelial cells from single transgenic Myc mice and implanted them into the

mammary fatpads of single transgenic TGF $\alpha$  mice from the same litter (derived from a heterozygote TGF $\alpha$ /Myc cross). This is a well known technique initially described by deOme (4). In this experiment we did not observe rapid onset mammary tumors (data not shown). We reasoned that one possible explanation was that TGF $\alpha$  expressed in high levels from the transgene in the mammary stroma of single transgenic TGF $\alpha$  mice was not actually secreted in high levels. As a first step in testing this hypothesis, we exploited single transgenic TGF $\alpha$  and bitransgenic TGF $\alpha$ /Myc mammary tumor cells of *in vitro* culture. We reverified that the cultures were expressing high levels of TGF $\alpha$  mRNA by northern blot and immunohistochemistry. However, in contrast to the TGF $\alpha$  cells, we were unable to detect secretion of significant levels of TGF $\alpha$  from bitransgenic cells in using a standard cloning bioassay for TGF $\alpha$  that we had previously described (2). These results were consistent with autocrine, but not paracrine action of TGF $\alpha$  in our system to allow interaction with Myc to promote tumors. The reasons for non-secretion of TGF $\alpha$  were not yet determined. Current studies are focused on repeating attempts to demonstrate paracrine effects of TGF $\alpha$  *in vivo* and on evaluation of the possibility of any immunologic barrier to transplantation.

## CONCLUSIONS

From these studies we have concluded the following relative to our year 1 specific aims:

1. TGF $\alpha$  and c-Myc interact in an extremely rapid fashion to induce the nearly simultaneous appearance of palpable tumors in males and females with an average latency of 66 days for each sex.
2. The time course for conversion of individual epithelial cells to full malignancy is extremely rapid, because disordered growth is apparent as early as the epithelium grows into the fatpad (3-4 weeks of age).
3. Testing of these early growing rudiments by transplantation subcutaneously into nude mice yielded invasive rapidly growing tumors. Thus, the TGF $\alpha$ /Myc interaction is profound, rapid, and seemly occurring too quickly for many further genetic changes to occur.
4. Surprisingly, although mammary tumors from single transgenic TGF $\alpha$  mice produced high levels of TGF $\alpha$  as determined by immunohistochemistry and northern blot, the protein did not appear to be secreted by the tumor cells in culture. This suggest that our proposed study to implant Myc - expressing epithelial cells into mice expressing a TGF $\alpha$  transgene in their stroma may fail. Specifically, stromal TGF $\alpha$  produced from the transgene may be cell- associated and unavailable for diffusion across the basement membrane to stimulate Myc - expressing epithelial cells. Indeed, in a pilot experiment where - Myc expressing mammary epithelial cells were implanted in TGF $\alpha$  - expressing stroma, mammary tumors failed to arise. Although additional attempts will be carried out for this experiment, it may be wise to concentrate further paracrine studies using our

second experimental design whereby single transgenic TGF $\alpha$  and Myc - expressing mammary epithelial cells are actually mixed prior to fatpad implantation. In this experimental design, juxtaposition contact could occur between TGF $\alpha$  and Myc cells to potentially allow tumorigenic synergy to occur.

From our studies, we were also able to make some unexpectedly rapid progress toward later year specific aims:

1. We were able to confirm expression of the TGF $\alpha$ , EGFR and Myc transgenes in bitransgenic tumors by northern blot and *in situ* hybridization. (See reference 3, included in the Appendix)
2. An unexpectedly clear secondary interaction of TGF $\alpha$  and Myc was also observed in salivary glands of male and female bitransgenic animals. Because these lesions were focal, rather than involving the entire gland (as with mammary lesions) we were able to utilize staining proliferating cell nuclear antigen (PCNA) to confirm, on a cellular level, the concordance of coexpression of TGF $\alpha$  and Myc with hyperproliferation and hyperplastic glandular morphology. (See reference 3 in Appendix)
3. Finally, because we were able to stockpile transgenic tumors from our study, with no added cost to the project, we were able to address expression of the TGF $\alpha$  - related growth factor amphiregulin. Surprisingly, this factor was strongly unregulated in all transgenic mouse mammary tumors, further supporting the role of the TGF $\alpha$  family of growth factors in mammary tumorigenesis (5).

## REFERENCES

1. Dickson, R.B.: The Molecular Basis of Breast Cancer in Kurzrock R and Talpaz M (eds) Molecular Biology in Cancer Medicine. Martin Dunitz, London, pp. 241-272, 1995.
2. Valerius, E.M., Ciardiello, F., Heldin, N.E., Blondel, B., Merlo, G., Smith, G., Stampfer, M.R., Lippman, M.E., Dickson, R.B., and Salomon, D.S.: Stromal influences on transformation of human mammary epithelial cells over expressing c-myc and SV40T. J. Cellular Physiology, 145:207-216, 1990.
3. Amundadottir, L.T., Johnson, M.D., Merlino, G.T., Smith, G.H., and Dickson, R.B.: Synergistic interaction of transforming growth factor  $\alpha$  and c-myc in mouse mammary and salivary gland tumorigenesis. Cell Growth and Differentiation. 6:737-748, 1995.
4. Daniel, C.W., Silberstein, G.B.: Postnatal development of the rodent mammary gland. In The Mammary Gland: Development, Regulation and Function. Neville, M.C. and Daniel, C.W. (eds.) New York: Plenum Press, pp. 3-36, 1987.
5. Kenny, N.J., Smith, G.H., Muller, W.J., Green, J., Callahan, R.H., Salomon, D.S., and Dickson, R.B.: Detection of amphiregulin and cripto-1 in mammary tumors from transgenic mice. Mol. Carcinogenesis, in press, 1995.

## Appendix

Amundadottir, L.T., Johnson, M.D., Merlino, G.T., Smith, G.H., and Dickson, R.B.: Synergistic interaction of transforming growth factor  $\alpha$  and c-myc in mouse mammary and salivary gland tumorigenesis. Cell Growth and Differentiation. 6:737-748, 1995.

# Synergistic Interaction of Transforming Growth Factor $\alpha$ and *c-myc* in Mouse Mammary and Salivary Gland Tumorigenesis<sup>1</sup>

Laufey T. Amundadottir, Michael D. Johnson, Glenn Merlino, Gilbert H. Smith, and Robert B. Dickson<sup>2</sup>

Vincent T. Lombardi Cancer Research Center [L. T. A., M. D. J., R. B. D.] and Department of Cell Biology [L. T. A., R. B. D.] Georgetown University, Washington, DC 20007, and Laboratories of Molecular Biology [G. M.] and Tumor Immunology and Biology [G. H. S.] National Cancer Institute, NIH, Bethesda, Maryland 20892

## Abstract

The *c-myc* oncogene is commonly amplified in breast cancer and is known to interact synergistically with transforming growth factor  $\alpha$  (TGF $\alpha$ ) *in vitro* to promote phenotypic transformation of mammary epithelial cells. In addition, both genes are under sex steroid hormone regulation in breast cancer. We have used a transgenic mouse approach to test the relevance of Myc-TGF $\alpha$  interaction in mammary gland tumorigenesis of virgin animals *in vivo*. We mated single transgenic TGF $\alpha$  and *c-myc* mouse strains to yield double transgenic offspring for TGF $\alpha$  and *c-myc*. All (20 of 20) double transgenic TGF $\alpha$ /*c-myc* animals developed synchronous mammary tumors at a mean age of 66 days. An unexpected finding was that tumor latency and frequency in males and virgin females were identical. Thus, two gene products that are known to be coinduced in breast cancer by the sex hormones estrogen and progesterone strongly synergize to induce synchronous mammary tumors, independent of sex. The tumors, despite being estrogen receptor positive, were readily transplanted as highly malignant s.c. cancers in ovariectomized nude mice. Although approximately one-half of single transgenic *c-myc* virgin females also eventually developed mammary gland tumors, these were stochastic and arose after a long latency period of 9–12 months. Single transgenic virgin TGF $\alpha$  females and males, *c-myc* males, and transgene-negative littermates did not develop tumors (ages up to 15 months).

The salivary glands of double transgenic animals also coexpress the two transgenes and show pathological abnormalities ranging from hyperplasias to frank adenocarcinomas. In contrast, the salivary glands of single transgenic and wild-type animals showed only mild hyperplasias or metaplasias, but tumors were not observed.

*In situ* hybridization analysis of mammary and salivary glands revealed that hyperplastic and tumorous areas colocalize with regions that overexpress both the TGF $\alpha$

and *c-myc* transgenes. This indicates that there is a requirement for the presence of both proteins for transformation of these glands. In summary, TGF $\alpha$  and *c-myc* synergize in an extremely powerful way to cause breast and salivary gland tumorigenesis in males and virgin females without a requirement for pregnancies.

## Introduction

Gene amplification and/or deregulated expression of a number of genes are frequent findings in human breast cancer. Among these are the genes for *c-myc* and TGF $\alpha$ .<sup>3</sup> The protein product of the *c-myc* gene is a nuclear phosphoprotein involved in transcriptional regulation, and TGF $\alpha$  is a member of the EGF family of mitogens, which bind to and activate the EGF receptor (1, 2). The *c-myc* proto-oncogene is amplified in 25 to 30% of breast cancer cases and is overexpressed (without gene amplification) in many more (3–6). Furthermore, amplification of the *c-myc* gene has been shown to correlate with poor prognosis of the disease (3, 7, 8). Although the TGF $\alpha$  gene is not found amplified in human breast cancer, its expression (and that of other EGF family members) is frequently increased compared to the normal gland (9–12). The EGFR is also found expressed in about 30–50% of human breast cancers with high expression associated with poor prognosis and high a degree of invasiveness (13).

Expression of both genes is induced during estrogen and progesterone treatment of hormone-responsive breast cancer cells *in vitro* (10, 14–18). In addition, treatment with antisense oligonucleotides to either TGF $\alpha$  or *c-myc* inhibits estrogen-induced expression of these genes and estrogen-stimulated growth *in vitro*, indicating that they are important mediators of estrogenic effects on cell growth (19, 20).

Transgenic mouse models have provided insight into the roles of both genes in mammary gland development and malignant progression *in vivo*. Overexpression of TGF $\alpha$  in the mammary gland from the mouse metallothionein promoter or the MMTV promoter/enhancer caused the appearance of mammary carcinomas after a relatively long latency period of 7–12 months. Tumors were stochastic and arose predominantly in female mice that had undergone multiple pregnancies (21–23). Transgenic mice with MMTV-*myc* constructs directing expression to the mammary gland also develop clonal tumors after a long latency period of 7–14 months, again with a requirement for multiple pregnancies (24).

Long latency times in transgenic mice are consistent with the hypothesis that oncogenesis is a multistage process composed of a series of genetic events (25, 26). Thus, although one proto-oncogene is overexpressed in a given

Received 12/28/94; revised 3/28/95; accepted 3/31/95.

<sup>1</sup> Supported by American Cancer Society Grant BE97B and Defense Department Grant DAMD17-94-J-4257 (to R.B.D.).

<sup>2</sup> To whom reprints for request should be addressed, at Lombardi Cancer Research Center, Room S-169, Georgetown University, 3800 Reservoir Road, NW, Washington DC 20007.

<sup>3</sup> The abbreviations used are: TGF $\alpha$ , transforming growth factor  $\alpha$ ; EGF, epidermal growth factor; EGFR, EGF receptor; MMTV, mouse mammary tumor virus; MT, metallothionein; LTR, long terminal repeat.

organ of a transgenic mouse, the occurrence of additional events is necessary before cancer can arise. We chose to study the interaction of TGF $\alpha$  with *c-myc*, since *in vitro* studies had suggested possible cooperative interactions. In various cell types *in vitro*, overexpression of *c-myc* resulted in an increased responsiveness to the effects of mitogenic growth factors. For example, rodent fibroblasts and human and mouse mammary epithelial cell lines transfected with *myc* constructs showed transformed behavior, in many cases, only in the presence of TGF $\alpha$  or fibroblast growth factor family members (27–30). High levels of *myc* expression may, therefore, permit a tumorigenic transformation by a TGF $\alpha$ /EGFR autocrine growth mechanism, or it may sensitize cells to such a mechanism.

The interaction of TGF $\alpha$  and *c-Myc* has not been studied in mammary gland transformation *in vivo*, but two recent studies investigated their interaction in the liver and pancreas with bitransgenic mouse models. Animals expressing both transgenes from liver- and pancreatic-specific promoters formed tumors in these organs at an elevated rate compared to single transgenic animals, suggesting a synergistic interaction (31, 32).

We show here that TGF $\alpha$  and *c-Myc* cooperate in an extremely powerful, synergistic manner in mouse mammary and salivary gland tumorigenesis. Double transgenic male and virgin female mice develop synchronous mammary tumors in all glands at about 2 months of age, whereas single transgenic animals develop clonal tumors at about 12 months of age or not at all. In addition, epithelial rudiments from 3-week-old TGF $\alpha$ /*c-myc* double transgenic animals could be established in nude mice, indicating that the mammary gland is transformed right at the start of its development. Tumors were also found in salivary glands of double transgenic animals at 3 months of age, whereas single transgenic and wild-type mice have not been observed to develop tumors. Expression of the TGF $\alpha$  and *c-myc* transgenes was associated with hyperplastic and tumorous areas in mammary and salivary glands, indicating a requirement for the presence of both gene products for malignant transformation.

## Results

**Generation of TGF $\alpha$ /*c-myc* Double Transgenic Mice.** Heterozygous mice transgenic for TGF $\alpha$  (MT-TGF $\alpha$  MT100 strain) (21) and *c-myc* (MMTV-*c-myc* M strain) (24) were mated to yield offspring of four possible groups: double transgenic TGF $\alpha$ /*c-myc* mice; single transgenic TGF $\alpha$  mice; single transgenic *c-myc* mice; and mice negative for transgenes (wild type). Since the parental mice are of FVB/N (TGF $\alpha$ ) and CD-1  $\times$  C57BL/6J (*c-myc*) backgrounds, offspring mice of all groups are of the following genetic background: FVBN/CD-1  $\times$  C57BL6J. Mice of the two single transgenic groups and of the wild-type group serve as controls in a similar genetic background as the double transgenic TGF $\alpha$ /*c-myc* mice.

At 3 weeks of age, offspring were weaned, and DNA was extracted from tail biopsies. Screening for transgenes was performed by Southern blot analysis and/or PCR (data not shown). According to Mendelian rules, when mating animals heterozygous for two traits, 25% of offspring should fall into each of four possible genotype groups. However, of 157 offspring, 45% were wild type; 15 and 25% were single transgenic for TGF $\alpha$  and *c-myc*, respectively; and 15% were double transgenic TGF $\alpha$ /*c-myc* (Table 1). Thus, there

Table 1 Number of offspring in each of four genotype groups

Mean mammary gland tumor onset times and tumor frequency in each group is presented.

Genotype	No. of animals (%) <sup>a</sup>	Mean tumor onset time (days)	Tumor incidence
TGF $\alpha$ / <i>c-myc</i>	23 (15)	66 $\pm$ 12	100% <sup>b</sup>
TGF $\alpha$	24 (15)	NA	0%
<i>c-myc</i>	39 (25)	298 $\pm$ 55	50%
Wild type	71 (45)	NA	0%

<sup>a</sup> Total offspring number is 157.

<sup>b</sup> One hundred % equals 20/20 animals; 3 animals died at very young ages. NA, not available (tumors were not observed for more than 1 year).

Table 2 Summary of histopathological findings in mammary glands of transgenic mice at 3 and 7 months of age

Genotype	3 mos.	7 mos.
TGF $\alpha$ / <i>c-myc</i>	Multiple adenocarcinomas (types A and B) in females and males <sup>a</sup>	NA
TGF $\alpha$	Normal	Cystic ducts
<i>c-myc</i>	Normal	Atypical hyperplasia to adenocarcinoma
Wild type	Normal	Cystic ducts

<sup>a</sup> Histopathology from both virgin females and males. All other diagnoses are from virgin females only. NA, not available (all animals of this genotype are deceased at this time point).

appeared to be a selection bias against mice positive for the TGF $\alpha$  transgene. A reduced body weight at weaning was not associated with the lower frequency genotypes (data not shown), in contrast to observations of Luetke *et al.* (33) for a different strain of MT-TGF $\alpha$  transgenic mice. However, we have noted that TGF $\alpha$ -positive mice consistently die at younger ages than *c-myc* single transgenic and wild-type mice and show signs of malnutrition. This is probably due to the effects of the TGF $\alpha$  transgene product on the stomach, as described previously (34). Offspring of each genotype group were approximately equally divided between females and males.

**Synergistic Induction of Mammary Gland Tumors in TGF $\alpha$ /*c-myc* Double Transgenic Mice.** Of 23 double transgenic TGF $\alpha$ /*c-myc* animals, 20 developed multiple mammary tumors at a mean age of 66  $\pm$  12 days, and three mice died from other causes at very young ages. We can, therefore, conclude that all mice of the TGF $\alpha$ /*c-myc* genotype that reached an age of about 2 months developed mammary gland cancers. An additional striking finding was that tumors arose in both virgin female and male animals with the same latency and frequency. Frank tumors (*i.e.*, palpable) arose first in axillary mammary glands (glands nos. 1, 2, and 3) and then subsequently in inguinal glands (nos. 4 and 5). The average number of palpable tumors at time of necropsy were 2.5/mouse. In addition, pathological diagnoses of hematoxylin/eosin-stained sections revealed the presence of adenocarcinomas in glands without frank palpable tumors, thus showing that every mammary gland from double transgenic animals was cancerous. Surprisingly, no normal tissue was found adjacent to mammary gland tumors in double transgenic animals; therefore, the whole gland could be characterized as malignant. Even a very young (5 weeks old) TGF $\alpha$ /*c-myc*-positive female was diagnosed as having



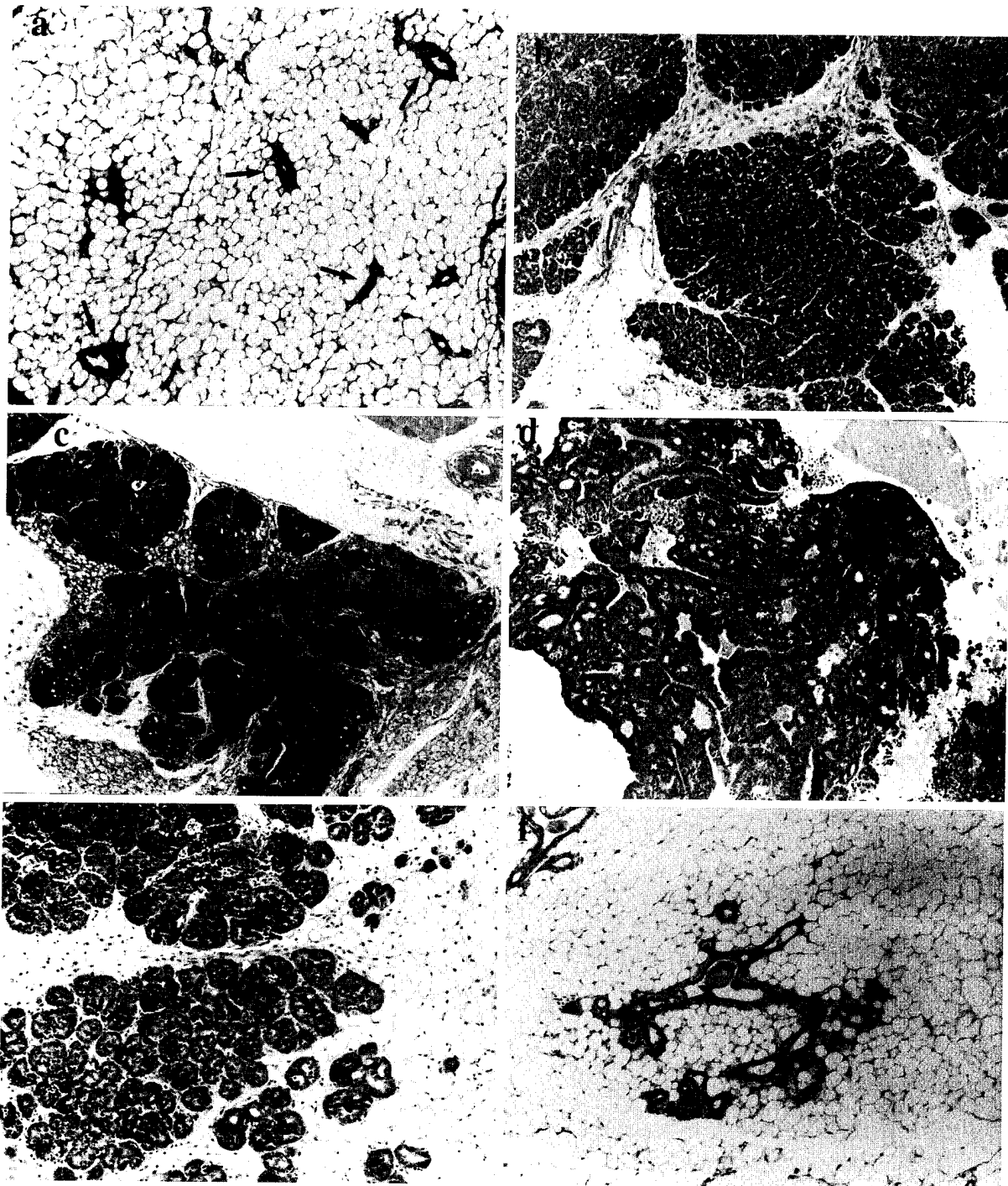


Fig. 1. Hematoxylin & eosin-stained sections of mammary glands. *a*, a normal virgin gland at 3 months of age. It is representative of TGf $\alpha$  and *c-myc* single transgenic animals and of nontransgenic littermates at this age. Arrows, epithelial ducts. Panels *b-d*, mammary gland tumors from double transgenic TGf $\alpha$ /*c-myc* animals at 3 months of age. *e*, a section of an inguinal mammary gland from a 5-week-old TGf $\alpha$ /*c-myc* female. *f*, an atypical hyperplastic gland from a *c-myc* single transgenic virgin female at 9 months of age.  $\times 100$ .

mammary gland adenocarcinoma based on histopathology. This is striking since, at this age, the gland is not fully developed. Our results suggest that, in this model, overexpression of TGf $\alpha$  and *c-myc* is sufficient to cause a com-

plete tumorigenic transformation of the mouse mammary gland.

Mean tumor onset times and frequency are shown in Table 1. Pathological diagnosis of mammary glands from

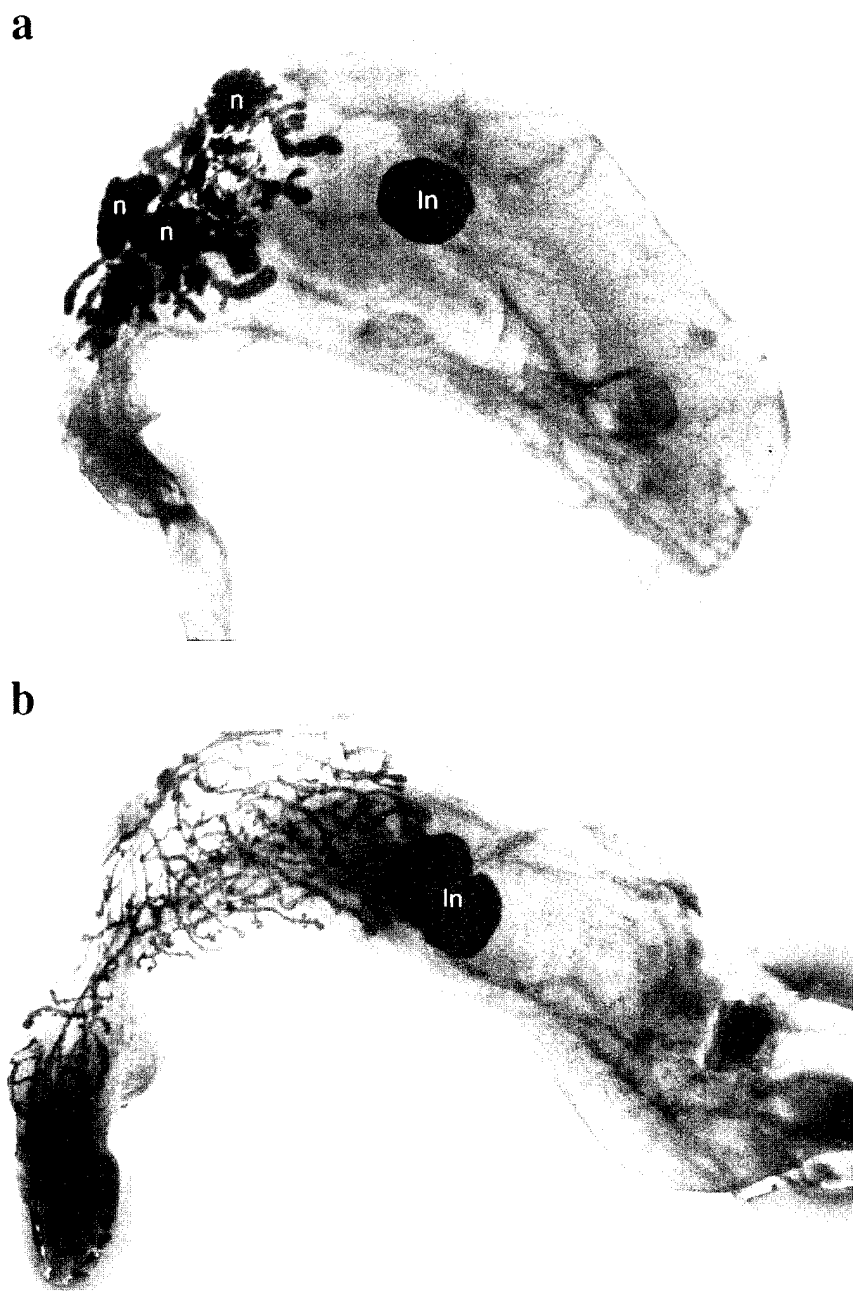


Fig. 2. Whole-mount staining of mammary glands from a TGF $\alpha$ /*c-myc* animal (a) and from a wild-type animal (b). The carmine alum stain reveals the epithelial network of the gland and the lymph node (*ln*). The animal in a was 24 days old, and the one in b was 28 days old. Note tumorous nodules (*n*) in the mammary gland from the TGF $\alpha$ /*c-myc* animal (a) and normal ductal pattern in the wild-type animal (b).

transgenic mice of each genotype group, at 3 and 7 months of age, is shown in Table 2. Representative hematoxylin/eosin-stained sections are shown in Fig. 1. The normal virgin gland at 3 months of age is mostly composed of adipose tissue, with scattered epithelial ducts consisting of two layers of cells (Fig. 1a). It is representative of pathology from the two single transgenic groups and the wild-type group at 3 months of age. In contrast, every mammary gland from double transgenic animals (virgin females and males) is tumorous at the same age (Fig. 1, b–d). Tumors were classified as adenocarcinomas of types A and B. A type tumors are fairly well differentiated, with the acinar struc-

ture of the gland prominent and two layers of epithelial cells seen surrounding lumens (Fig. 1b). Type B is less organized and locally invasive (Fig. 1d). When tumor sections were stained with periodic acid-Schiff stain, the basement membrane was seen intact in type A tumors but was often disrupted in type B tumors (data not shown). No distant metastases have been found to date. Fig. 1e shows adenocarcinoma from a 5-week-old double transgenic virgin female animal. At this age, the epithelial tree has not fully penetrated the mammary fat pad.

About 50% single transgenic *c-myc* virgin females also developed mammary gland tumors, but these were stochas-

tic and arose only after a very long latency period of  $298 \pm 55$  days. The remainder had mild atypical hyperplasias and cystic ducts (Fig. 1f). Single transgenic virgin TGF $\alpha$  mice and transgene-negative littermates have not developed tumors to this date (ages up to 15 months). In the case of single transgenic males, we observed atypical hyperplastic areas in mammary glands of a 14-month-old single transgenic *c-myc* male (data not shown). Mammary glands from single transgenic TGF $\alpha$  males and wild-type males at the same ages were normal.

A whole organ staining (termed whole-mount staining) of mammary glands from virgin double transgenic animals at 24 days of age revealed multiple nodules in each gland that appeared tumorous (Fig. 2a). These were successfully established in nude mice, indicating that the gland is transformed from the start of its development. In comparison, whole-mount staining of mammary glands from wild-type virgin animals at 28 days of age revealed only the normal ductal pattern (Fig. 2b).

The observation that mammary gland tumors arose in double transgenic TGF $\alpha$ /*c-myc* males as well as in virgin females suggested that they might be estrogen independent. Estrogen receptor ligand binding assays revealed that tumors from males and females contained from 13–30 fmol/mg protein of the receptor, and are, therefore, considered estrogen receptor positive (data not shown). Control tumors (MCF-7 or MKL-4 cells grown as tumors in nude mice) contained about 3-fold higher levels of receptor. The ovariectomizing of TGF $\alpha$ /*c-myc* females ( $n = 2$ ) at the time of weaning did not result in a significantly delayed tumor onset (69 versus 66 days). In addition, both axillary and inguinal mammary gland tumors could be successfully transplanted into ovariectomized nude mice (data not shown). Together, these data indicate that, although relatively low levels of the estrogen receptor are present in mammary gland tumors as measured by binding to ligand, they are not dependent on estrogen for growth.

**Expression of Transgenes and the Epidermal Growth Factor Receptor in Mammary Gland Tumors.** We have used Northern analysis, RNase protection assays, *in situ* hybridization analysis, and immunohistochemistry to examine the expression of transgenes and that of the EGFR gene in mammary gland tumors from double transgenic TGF $\alpha$ /*c-myc* animals. RNA expression was compared between axillary mammary gland tumors (frank tumors or lumps) and inguinal gland tumors (carcinoma revealed by histopathology) of TGF $\alpha$ /*c-myc* animals.

Transgenes were expressed in all mammary gland tumors from double transgenic animals but were not detectable in normal glands from single transgenic animals at 3 months of age (Fig. 3). There was about a 5-fold difference in the expression of the *c-myc* transgene, and about 7-fold for the TGF $\alpha$  transgene between the lowest- and highest-expressing tumor. An association of transgene expression at the RNA level and pathological diagnosis (adenocarcinoma type A versus B), location (axillary versus inguinal glands), tumor size, or sex was not observed. Expression of TGF $\alpha$  and *c-myc* was not detected in normal glands by this method. Transgene expression was also examined by *in situ* hybridization analysis to establish the pattern of transgene expression in the tumors (Fig. 4, a, c, and e). Sequential tumor sections from double transgenic TGF $\alpha$ /*c-myc* animals were hybridized to  $^{35}$ S-labeled TGF $\alpha$  and *c-myc* probes. We observed a very strong and uniform expression of *c-myc* mRNA in mammary gland tumors from double

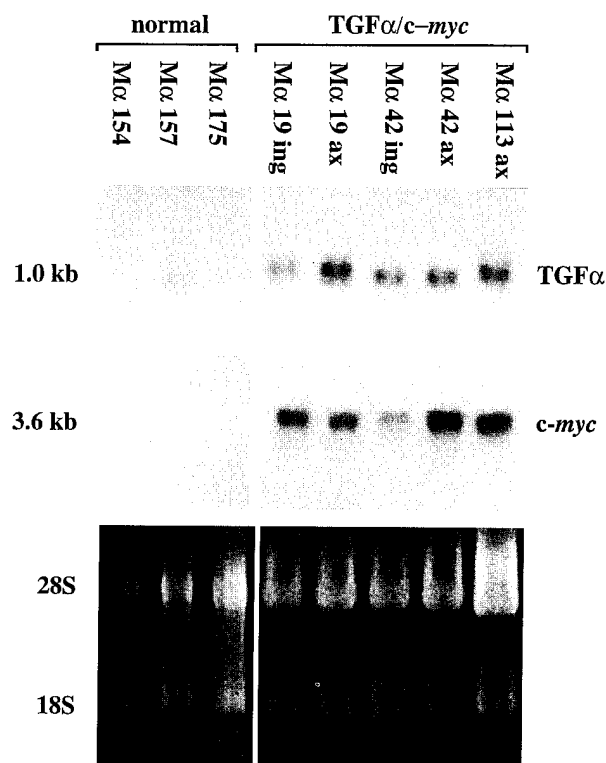


Fig. 3. Northern analysis showing expression of the TGF $\alpha$  and *c-myc* transgenes in mammary gland tumors from double transgenic animals and in normal glands from single transgenic and wild-type animals. The TGF $\alpha$  transcript is 1.0 kb, and the *c-myc* transcript is 3.6 kb. Note that the endogenous 2.3-kb *c-myc* transcript is not seen here. Loading controls are the 18S and 28S ribosomal RNA bands. M $\alpha$  19 through 175 denotes the number of each animal used here. Ax, axillary gland tumor; ing, inguinal gland tumor.

transgenic animals at 3 weeks of age and higher. A scattered expression of TGF $\alpha$  mRNA was noted in most areas of the mammary glands by this method. An immunohistochemical evaluation of TGF $\alpha$  protein levels in bitransgenic tumors with a TGF $\alpha$ -specific antibody also revealed a scattered pattern of strong expression but no association of enhanced staining with a more aggressive phenotype (Fig. 5, a and b). We also measured endogenous EGFR mRNA levels in tumors from double transgenic animals by RNase protection assays. As seen in Fig. 6, EGFR mRNA levels were comparable in all but one mammary gland tumor (axillary tumor from TGF $\alpha$ /*c-myc* animal no. 113). An association of EGFR mRNA levels with pathological diagnosis, location, tumor size, or sex was not seen.

**Synergistic Induction of Salivary Gland Tumors by TGF $\alpha$  and *c-myc*.** The MT promoter is active in most epithelial tissues, whereas the MMTV promoter is restricted to only a few tissues. Therefore, the MMTV promoter limits coexpression of the transgenes to mammary glands, salivary glands, and some reproductive organs. An interaction between TGF $\alpha$  and *c-myc* was not observed in reproductive organs, but a positive interaction was noted in the salivary glands. Ductule hyperplasia (sometimes with atypia) was seen in all salivary glands of double transgenic TGF $\alpha$ /*c-myc* virgin female and male animals at 3 months of age. In some cases, squamous metaplasia was observed in the sublingual gland,

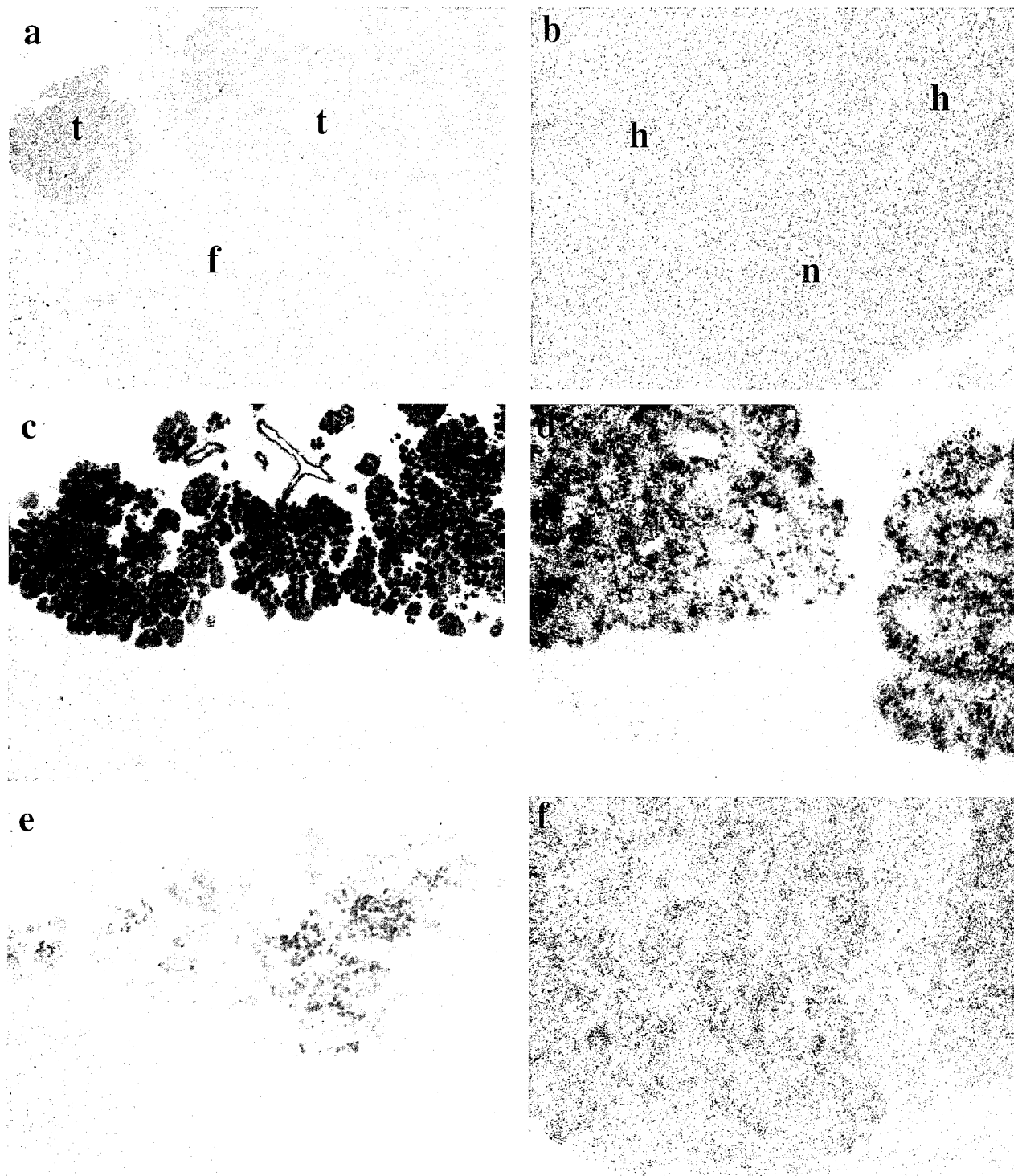


Fig. 4. *In situ* hybridization analysis of mammary and salivary glands from TGF $\alpha$ /*c-myc* animals. Sequential paraffin-embedded tissue sections were hybridized to  $^{35}$ S-labeled riboprobes generated against the TGF $\alpha$  and *c-myc* transgenes. *a* and *b*, control sections hybridized to sense riboprobes. *c* and *d*, sections hybridized to *c-myc* antisense riboprobes. *e* and *f*, sections hybridized to TGF $\alpha$  antisense riboprobes. *a*, *c*, and *e*, from mammary glands; *b*, *d*, and *f*, from salivary glands. Note a near uniform expression of the transgenes in a mammary gland tumor from this 3-week-old double transgenic animal. Observe a patchy expression of *c-myc* and scattered distribution of TGF $\alpha$  in this 6-week-old double transgenic animal. Also note that where both transgenes are expressed, the glands appear hyperplastic and tumorous. *t*, tumor; *f*, fatty tissue; *h*, hyperplastic; *n*, normal. All panels are brightfield photographs. *a*, *c*, and *e*,  $\times 50$ ; *b*, *d*, and *f*,  $\times 200$ .

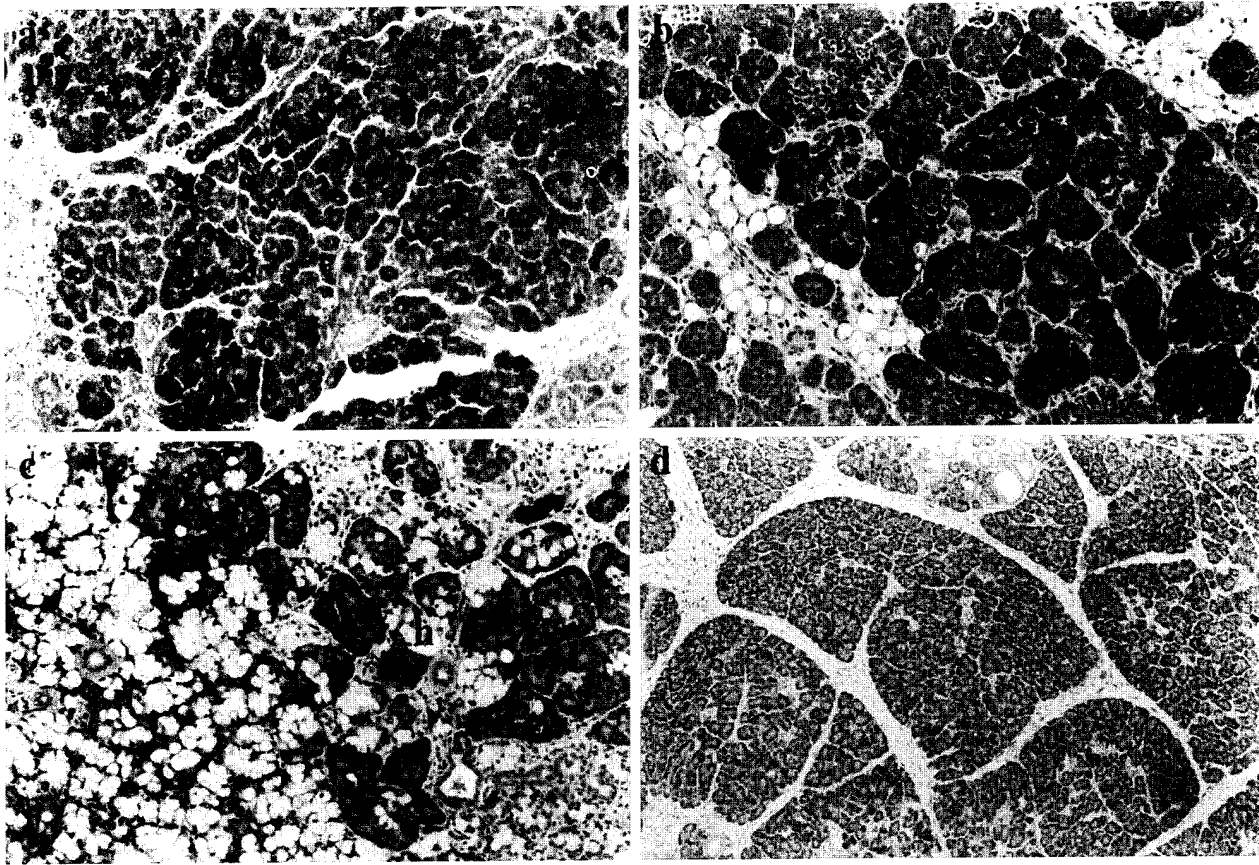


Fig. 5. Immunohistochemical staining of mammary and salivary glands from TGF $\alpha$ /c-myc double transgenic animals with an anti-TGF $\alpha$  polyclonal antiserum. a, a mammary gland tumor from a 3-month-old animal and b, from a 5-week-old animal. Both have a scattered pattern of strong TGF $\alpha$  staining. c, an immunohistochemical staining of a bitransgenic salivary gland (sublingual) with a premalignant atypical hyperplastic lesion (h) that stains strongly for TGF $\alpha$ , while the surrounding normal areas (n) appear negative. d, a negative control without a primary antibody.

and adenoma and adenocarcinoma in the parotid gland at the same age. Salivary glands of single transgenic TGF $\alpha$  animals showed minimal ductule hyperplasia, but single transgenic c-myc mice and transgene-negative littermates were free of pathological abnormalities at 3 months of age (Table 3; Fig 7).

At 7 months of age, histopathology of salivary glands from single transgenic animals revealed minimal serous metaplasia in the sublingual glands of c-myc mice, and mild duct(ule) squamous metaplasia and hyperplasia in the sublingual and submandibular glands of TGF $\alpha$  animals. Wild-type mice at 7 months of age had no apparent abnormalities. No tumors were ever observed in the salivary glands of single transgenic or wild-type mice (up to 10 months for TGF $\alpha$  mice and 15 months for c-myc mice).

To obtain information about the localization of expression of both transgenes within the gland, *in situ* hybridization analysis was performed on sequential sections of salivary glands from TGF $\alpha$ /c-myc animals (Fig. 4, b, d, and f). It revealed a very patchy pattern of expression of the c-myc transgene and scattered expression of the TGF $\alpha$  transgene. Expression was quite different from what we observed in the mammary glands in that only about 5% of salivary gland tissue was positive for both transgenes. Interestingly, areas where expression of both transgenes

was detected appeared hyperplastic and atypical, whereas areas with only one transgene expressed looked quite normal. These areas might represent premalignant areas within the salivary glands, indicating that only when both transgenes are expressed does malignant conversion occur. Immunohistochemical staining of salivary glands from TGF $\alpha$ /c-myc animals was performed with a polyclonal antiserum that recognizes both the endogenous mouse TGF $\alpha$  and the transgene-derived human TGF $\alpha$ . In agreement with *in situ* hybridization data, we observed a scattered distribution of TGF $\alpha$  expression and an association of an intense staining with premalignant hyperplastic atypical nodules (as seen in the sublingual gland in Fig. 5c), whereas surrounding areas of normal or hyperplastic salivary gland had little or no TGF $\alpha$  staining. Finally, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) on salivary gland sections revealed a strong staining in areas that coexpressed the transgenes, indicating that DNA synthesis was occurring (Fig. 8).

### Discussion

In this study, matings of MT-TGF $\alpha$  and MMTV-c-myc transgenic strains were carried out to investigate the *in vivo*

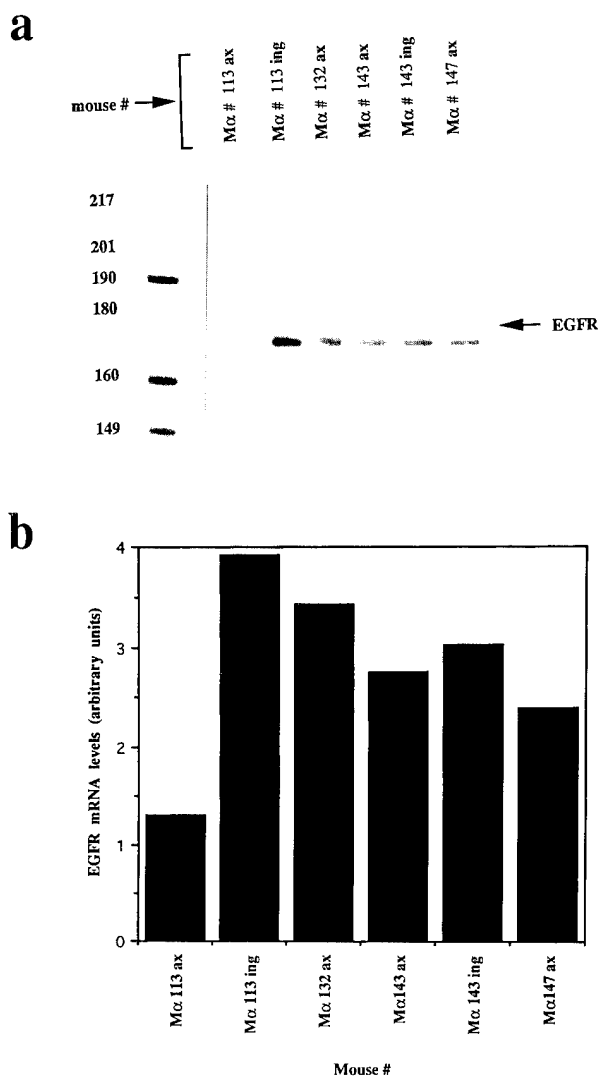


Fig. 6. Expression of the endogenous EGFR mRNA measured by RNase protection assay. RNA levels were compared between mammary gland tumors from double transgenic animals. *b*, a scanned version of the data in *a*. Mα113 through Mα147 denotes the number of each double transgenic mouse used in the assay. ax, axillary gland tumor; ing, inguinal gland tumor.

interaction of TGF $\alpha$  and c-Myc in mouse mammary gland transformation and confirm our previous *in vitro* studies that showed cooperation between the two. We found that tumorigenesis in mammary glands of double transgenic TGF $\alpha$ /c-myc virgin females and males is strikingly different from single transgenic and wild-type animals and also from multiparous single transgenic TGF $\alpha$  and c-myc mice. In previous studies, both MT-TGF $\alpha$  and MMTV-c-myc single transgenic mice developed polyclonal mammary gland tumors only after a long latency period and multiple pregnancies (21, 24, 35). In contrast, our studies show that both virgin females and males harboring both transgenes develop multiple mammary gland tumors after a mean latency period of only 66 days. All of 20 double transgenic virgin females and males developed rapidly growing mammary gland tumors that could be established in nude mice in the absence of estrogens. Single transgenic virgin TGF $\alpha$  and

Table 3 Summary of histopathological findings in salivary glands of transgenic animals at 3 and 7 months of age

All data represent both male and female animals.		
Genotype	3 mos.	7 mos.
TGF $\alpha$ /c-myc	Hyperplasia with atypia, squamous metaplasia and adenoma, and adenocarcinoma	NA <sup>a</sup>
TGF $\alpha$	Ductule hyperplasia	Ductule hyperplasia and squamous metaplasia
c-myc	Normal	Serous metaplasia
Wild type	Normal	Normal

<sup>a</sup> NA, not available (all animals of this genotype are deceased at this time point).

wild-type animals of both sexes did not develop any tumors, whereas about one-half single transgenic c-myc virgin females developed stochastic mammary gland tumors after a long latency period of about 8–12 months. The early onset and multiple tumor formation in double transgenic TGF $\alpha$ /c-myc animals suggests that very few, if any, additional genetic events are necessary for tumorigenesis in our model. In fact, at 3 weeks of age, when the glandular tissue has just started to penetrate the fat pad, the mammary gland is already tumorous.

It is also quite interesting that tumors form in a synchronous manner in our model, so that normal mammary gland tissue is not found at all. Two previous studies have described transgenic models with synchronous tumorigenesis of mammary glands. In the first one, an activated rat *neu* oncogene was expressed from the MMTV-LTR promoter/enhancer, and in the second, the polyoma middle T oncogene was expressed from the same promoter (36, 37). An extremely high level of transgene expression observed in the former strain might have contributed to the phenotype, since MMTV-*neu* transgenic mice made by another group developed only stochastic mammary gland tumors (38). However, the study has been repeated using the same transgene construct with similar results (39). Our model is comparable to the effects of a mutated growth factor receptor, Neu, or the powerful viral protein product of the polyomavirus middle T oncogene that mediates cellular transformation by targeting a number of intracellular signaling pathways (40–43). The fact that overexpression of two normal proteins in the mammary gland of transgenic mice has a similar effect on tumorigenesis in this organ as a mutated, highly active growth factor receptor and a strong viral oncoprotein further emphasizes the cooperative effect of TGF $\alpha$  and c-Myc.

An intriguing finding from our studies was that double transgenic TGF $\alpha$ /c-myc males developed mammary cancer in a manner indistinguishable from virgin females. Mammary gland cancer has been described previously in transgenic male mice of MMTV-*neu*, MMTV-v-Ha-ras, MMTV-*int-1*, MMTV-*int-3*, and MMTV-polyomavirus middle T strains. However, tumor onset is typically delayed compared to female mice (35–37, 44, 45). Both TGF $\alpha$  and c-myc are estrogen-inducible genes, and each has been shown to be responsible, at least in part, for estrogen-mediated growth *in vitro* (19, 20). It is, therefore, possible that when both genes are overexpressed *in vivo*, they induce growth of the male mammary gland in the absence of



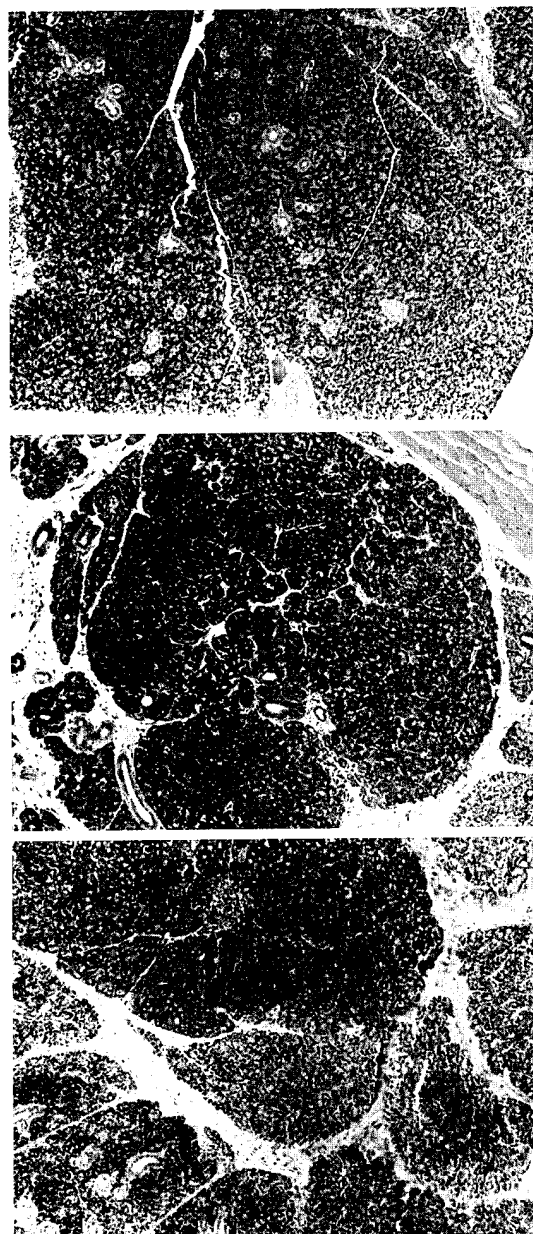


Fig. 7. Hematoxylin and eosin-stained sections of salivary glands. *a*, a normal parotid gland from a wild-type mouse. *b*, a parotid adenoma and *c*, an adenocarcinoma from TGF $\alpha$ /c-myc double transgenic animals. All animals are about 3 months old.  $\times 100$ .

estrogens. Breast cancer in human males is extremely rare, but in the presence of exogenously applied estrogens, males can develop mammary gland hyperplasias (gynecomastia), thus showing that estrogens can cause proliferation in the male mammary gland in the presence of androgens. It is remarkable that overexpression of two estrogen-induced genes can stimulate the growth and malignant transformation of the male mammary gland as we have seen here. It encourages further studies to dissect the role of, and interaction between, mediators responsible for hormone action on the normal and malignant development of the mammary gland.

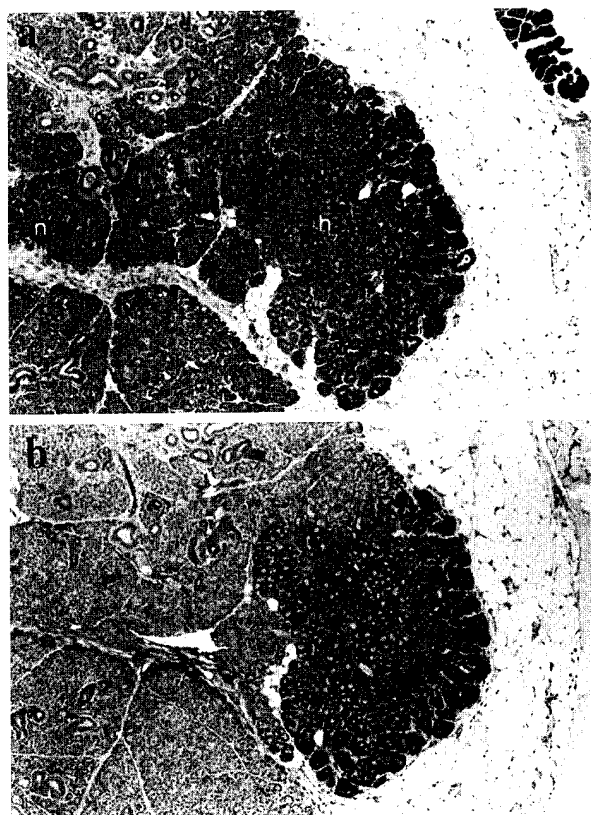


Fig. 8. Immunohistochemical staining of proliferating cell nuclear antigen in salivary glands from a 6-week-old double transgenic TGF $\alpha$ /c-myc animal. *a*, a hematoxylin and eosin-stained section of the salivary gland and *b*, a sequential section stained for proliferating cell nuclear antigen. Note intense proliferating cell nuclear antigen staining in hyperplastic areas of this field. This area in the parotid gland coexpressed the TGF $\alpha$  and c-myc transgenes. *h*, hyperplastic; *n*, normal.  $\times 100$ .

A cooperative interaction between TGF $\alpha$  and c-Myc also exists in the salivary glands, although malignancies were somewhat less prominent than in mammary glands. In contrast to mammary glands of bitransgenic mice where normal tissue was not found, the salivary glands of double transgenic animals contained normal tissue juxtaposed with hyperplastic areas and frank tumors. However, single transgenic and wild-type animals did not develop salivary gland tumors, whereas they developed mammary gland tumors after a long latency (MMTV-c-myc female mice). Therefore, we conclude that a strong cooperative interaction also exists in the salivary glands.

*In situ* hybridization analysis revealed that expression of transgenes was more uniform in mammary glands than salivary glands. In fact, only about 5–10% of salivary gland tissue expressed detectable levels of c-Myc (mainly in the parotid gland). Interestingly, areas that expressed both transgenes appeared hyperplastic (salivary glands at 5 weeks) or tumorous (mammary glands at 3 weeks), indicating a requirement for both TGF $\alpha$  and c-Myc in tumorigenesis. In the salivary gland, this was quite clear since areas were found that expressed either TGF $\alpha$ , c-myc, both transgenes, or no transgenes. A progressive tumor onset was associated with a patchy expression of transgenes in the salivary glands. On the other hand, an extremely rapid

tumor onset was associated with a near uniform expression of transgenes in the mammary glands. This might suggest that additional events must occur in the course of the slower tumorigenesis in salivary glands. In the mammary glands, TGF $\alpha$  and *c-Myc* appear to be sufficient to mediate transformation, although additional events cannot be ruled out. In both glands, there appears to be a selective advantage to express increasing levels of the TGF $\alpha$  transgene in the course of malignant progression. The mechanism of this effect is not known, but an apparently similar phenomenon was observed previously in mouse skin carcinogenesis in transgenic TGF $\alpha$  mice (46).

In summary, TGF $\alpha$  and *c-myc* are extremely powerful, synergistic-acting genes in breast and salivary gland carcinogenesis in the mouse strains described here. Since TGF $\alpha$  and *c-Myc* cause uniform transformation of the mammary gland of transgenic mice, this model provides an ideal system to examine possible secondary events for malignant progression/metastasis and characterize the relevance of a deregulated TGF $\alpha$ /EGF receptor pathway in mammary tumorigenesis.

## Materials and Methods

**Transgenic Mice.** The MMTV-*c-myc* mice used in this study were obtained from Charles River Laboratories (Wilmington, MA); experiments were carried out under a breeding license agreement with Du Pont Medical Products (Wilmington, DE). All mice were rederived and were free of adventitious agents. Line MT100 has a mouse metallothionein I (MT) promoter driving expression of a human TGF $\alpha$  cDNA transgene in an FVB/N inbred genetic background (21). The MMTV-*c-myc* M line harbors a mouse *c-myc* gene driven by the mouse mammary tumor virus long terminal repeat promoter/enhancer (MMTV-LTR) in a CD-1  $\times$  C57BL/6J background (24). Both strains have been shown to form stochastic mammary gland tumors after a long latency period and multiple pregnancies. Double transgenic mice were generated by mating the MT-TGF $\alpha$  MT100 line to the MMTV-*c-myc* M line. Offspring were maintained on 50 mM ZnCl<sub>2</sub> drinking water from the time of weaning (3–4 weeks of age) to induce maximal expression of the TGF $\alpha$  transgene from the metallothionein promoter.

**Genotyping of Offspring for Transgenes.** DNA was isolated from 1-cm tail biopsies by an overnight proteinase K digestion at 55°C, followed by phenol/chloroform extractions and ethanol precipitation. For Southern analysis, 10  $\mu$ g of tail DNA was digested overnight at 37°C with the following restriction enzymes: *Bgl*II for TGF $\alpha$ ; and *Bam*HI and *Cl*al for *c-myc*. After electrophoresis through 0.8% agarose gels and transfer to nitrocellulose, blots were probed with random-primed <sup>32</sup>P-labeled cDNA probes for human TGF $\alpha$  and mouse *c-myc*. The TGF $\alpha$  probe was a 925-bp *Eco*RI fragment from the plasmid pTGF $\alpha$ , kindly provided by Dr. Francis Kern (Georgetown University, Washington, DC; Ref. 47). The *c-myc* probe was a 2400-bp *Eco*RI-*Xba*I fragment from the plasmid fpGV-1, generously provided by Dr. MaryLou Cutler (NIH, Bethesda, MD; Ref. 48).

For PCR, 3  $\mu$ g DNA from tail biopsies was used as a template to amplify transgenes. 3' primers were complementary to sequences in the TGF $\alpha$  and *c-myc* transgenes, and 5' primers to sequences in the metallothionein (MT) and MMTV promoters, respectively: MT-TGF $\alpha$  5' primer, 5'-TCG TCC CCG AGC CAG TCG-3'; MT-TGF $\alpha$  3' primer,

5'-GTC CGT CTC TTT GCA GTT CTT-3'; MMTV-*c-myc* 5' primer, 5'-CCC AAG GCT TAA GTA AGT TTT TGG-3'; and MMTV-*c-myc* 3' primer, 5'-GGG CAT AAG CAC AGA TAA AAC ACT-3'. Primers were made by the Lombardi Cancer Center Macromolecular Synthesis and Sequencing Core facility (Georgetown University, Washington DC). PCR was performed using the Perkin Elmer Taq polymerase kit (Perkin Elmer, Norwalk, CT). The TGF $\alpha$  and *c-myc* transgenes were detected with Southern analysis and/or PCR.

**Tumors and Histopathology.** Mice were palpated bi-weekly for tumors and sacrificed before tumor sizes reached 10% of body weight. Location and size of each tumor were determined. Tumors were fixed in Bouin's solution for 5–12 h, embedded in paraffin, sectioned at 5  $\mu$ m, stained with hematoxylin and eosin, and examined to determine histopathological diagnoses. Transplantation of tumors into ovariectomized female NCR *nu/nu* mice was performed as follows. Tumor-bearing mice were anesthetized with methofane, and tumors were excised aseptically. Tumors were cut into about 1-mm<sup>2</sup> pieces and inserted s.c. (between nipples nos. 2 and 3) of nude mice under anesthesia.

**Whole-Mount Staining.** Animals were sacrificed, and the inguinal mammary glands were removed and fixed in 25% glacial acetic acid and 75% ethanol for 60 min at room temperature. After staining overnight in carmine alum solution [1 g carmine and 2.5 g aluminium potassium sulfate (both from Sigma Chemical Co.) in 500 ml water] glands were dehydrated in a series of ethanol washes and finally cleared in toluene. Glands were stored and photographed in methyl salicylate.

**Estrogen Receptor Binding Assay.** Frozen tumor samples (50–100 mg) were pulverized in liquid nitrogen and homogenized at 0°C in TEDG [10 mM Tris-OH (pH 7.4), 1 mM EDTA, 1 mM DTT, and 10% glycerol] plus 0.5 M NaCl and a cocktail of proteolysis inhibitors (leupeptin at 1 mg/ml, aprotinin at 77  $\mu$ g/ml, and pepstatin A at 1  $\mu$ g/ml). Homogenates were centrifuged at 105,000  $\times$  g at 4°C for 30 min to yield a whole-cell lysate, which was then adjusted to 2 mg/ml protein. Lysates were incubated with 10 nM [<sup>3</sup>H]17 $\beta$ -estradiol with or without a 100-fold excess of unlabeled estradiol for 16 h at 4°C. Binding was assayed by adding dextran-coated charcoal to adsorb free hormone. After centrifugation, aliquots of supernatant were removed and counted in 10 ml of liquid scintillation fluid in a Beckman liquid scintillation counter. Estrogen receptor-positive control tumors were MCF-7 and MKL-4 breast cancer cell lines grown in nude mice (49). They were generously provided by Dr. Sandy McLeskey (Georgetown University, Washington DC).

**RNA Isolation and Analysis.** Total RNA was isolated by pulverizing frozen tumors in liquid nitrogen, followed by homogenization in guanidine thiocyanate, acid phenol extraction, and precipitation with isopropanol. TGF $\alpha$  and *c-myc* transgene expression was assessed by Northern blot hybridization; 15  $\mu$ g total RNA were electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Amersham, Arlington Heights, IL), and probed with a <sup>32</sup>P-labeled random-primed probes. The *c-myc* probe was generated from the plasmid fpGV-1 (as described above) and the TGF $\alpha$  probe from the plasmid pTGF $\alpha$ -RP as described previously (50).

EGFR expression was determined using RNase protection assays in which <sup>32</sup>P-labeled antisense riboprobes (cRNA) were synthesized *in vitro* from the plasmid pME2.0 for the



EGFR, by linearizing with *Hind*III and transcribing with SP6 polymerase (51). It yields a 170-bp protected EGFR fragment. This plasmid was kindly provided by Dr. M. Rosner (University of Chicago, Chicago, IL). Total RNA (30 µg; EGFR) was hybridized for 12–16 h at 42°C to the <sup>32</sup>P-labeled cRNA probe and treated with RNase A for 30 min at 25°C. The radiolabeled riboprobes protected by total RNA were run on a 6% polyacrylamide/7M urea gel, which was subsequently dried and exposed to autoradiography.

**In Situ Hybridization Analysis.** To detect localization of transgene expression, *in situ* hybridization analysis was performed on mammary and salivary glands from 3; 6; and 10-week old double transgenic TGFα/c-myc animals. Animals were sacrificed, and glands were fixed in 4% paraformaldehyde in PBS for 24 h. *In situ* hybridization analysis was performed by Molecular Histology, Inc. (Gaithersburg, MD Ref. 52). Probes were generated from plasmids; p.c.-myc20 was generously provided by Dr. S. Thorgeirsson (NIH, Bethesda, MD). For the antisense riboprobe, this plasmid was linearized with *Eco*RI and transcribed with T7 polymerase. For a sense control riboprobe, the same plasmid was linearized with *Hind*III and transcribed with SP6 polymerase. Plasmid pTGFα-pGem3Z was used to detect the TGFα transgene. For an antisense riboprobe, this plasmid was linearized with *Hind*III and transcribed with T7 polymerase. For a sense control riboprobe, the same plasmid was digested with *Eco*RI and transcribed with SP6 polymerase.

**Immunostaining.** Tissues were fixed in Bouin's solution for 5–12 h, embedded in paraffin, and sectioned. After treatment with 0.02% trypsin for 15 min, sections were incubated overnight at room temperature with a 1:20,000 dilution of a rabbit polyclonal antiserum generated against a rat pro-TGFα intracellular peptide (residues 137–159). The antibody was kindly provided by Dr. Larry Gentry (Medical College of Ohio, Toledo, OH). TGFα was localized using the Vectastain Rabbit Elite kit (Vector Laboratories, Burlingame, CA), as described previously (50). PCNA immunostaining was performed as described previously (53).

## Acknowledgments

We thank Dr. Philip Leder for providing the c-myc transgenic strain. We also thank Drs. Miriam Anvers, Soon Paik, and Elizabeth Montgomery for tumor diagnoses; Marybeth Sabol, Daniel Will, and Thomas Carpenter for assistance with animal maintenance; Dr. Michio Maemura for help with animal necropsies; and Dr. Dorraya El-Ashry for help with estrogen receptor ligand binding assays. Finally, we thank Ann Wright, Kimberly Wingate-Legette, and Ronald Walden and other staff at the Research Resource Facility, Georgetown University, for invaluable help with animal experiments.

## References

- Luetke, N. C., and Lee, D. C. Transforming growth factor α: expression, regulation and biological action of its integral membrane precursor. *Semin. Cancer Biol.*, 1: 265–276, 1990.
- Meichle, A., Philipp, A., and Eilers, M. The functions of the Myc proteins. *Biochim. Biophys. Acta*, 1114: 129–146, 1992.
- Bonilla, M., Ramirez, M., Lopez-Cueto, J., and Gariglio, P. *In vivo* amplification and rearrangement of c-myc oncogene in human breast tumors. *J. Natl. Cancer Inst.*, 80: 665–671, 1988.
- Escot, C., Theillet, C., Liderau, R., Spyrtos, F., Champeme, M., Gest, J., and Callahan, R. Genetic alteration of the c-myc protooncogene (MYC) in human primary breast carcinomas. *Proc. Natl. Acad. Sci. USA*, 83: 4834–4838, 1986.
- Garcia, I., Dietrich, P. Y., Aapro, M., Vauthier, G., Vadas, L., and Engel, F. Genetic alteration of c-myc, c-erbB-2 and c-Ha-ras protooncogenes and clinical associations in human breast carcinomas. *Cancer Res.*, 49: 6675–6679, 1989.
- Mariani-Costantini, R., Escot, C., Theillet, C., Gentile, A., Merlo, G., Liderau, R., and Callahan, R. *In situ* c-myc expression and genomic status of the c-myc locus in infiltrating ductal carcinomas of the breast. *Cancer Res.*, 48: 199–205, 1988.
- Borg, A., Baldetorp, B., Ferno, M., Olsson, H., and Sigurdsson, H. c-myc amplification is an independent prognostic factor in postmenopausal breast cancer. *Int. J. Cancer*, 51: 687–691, 1992.
- Tavassoli, M., Quirke, P., Farzaneh, F., Lock, N. J., Mayne, L. V., and Kirkham, N. c-erbB-2/c-erbA co-amplification indicative of lymph node metastasis, and c-myc amplification of high tumor grade, in human breast carcinoma. *Br. J. Cancer*, 60: 505–510, 1989.
- Arteaga, C. L., Hanauske, A. R., Clark, G. M., Osborne, K., Hazarika, P., Pardue, R. L., Tio, F., and Von Hoff, D. D. Immunoreactive α transforming growth factor activity in effusions from cancer patients as a marker of tumor burden and patient prognosis. *Cancer Res.*, 48: 5023–5028, 1988.
- Bates, S. E., Davidson, N. E., Valverius, E. M., Freter, C. E., Dickson, R. B., Tam, J. P., Kudlow, J. E., Lippman, M. E., and Salomon, D. S. Expression of transforming growth factor α and its messenger ribonucleic acid in human breast cancer: its regulation by estrogen and its possible functional significance. *Mol. Endocrinol.*, 2: 543–553, 1988.
- Derynck, R., Goeddel, D. V., Ullrich, A., Gutterman, J. U., Williams, R. D., Bringman, T. S., and Berger, W. H. Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. *Cancer Res.*, 47: 707–712, 1987.
- Travers, M. T., Barrett-Lee, P. J., Berger, U., Luqmani, Y. A., Gazet, J. C., Powles, T. J., and Coombes, R. C. Growth factor expression in normal, benign, and malignant breast tissue. *Br. Med. J.*, 296: 1621–1624, 1988.
- Harris, A. L., and Nicholson, S. Epidermal growth factor receptors in human breast cancer, p. 93–118. In: M. E. Lippman and R. B. Dickson (eds.), *Breast Cancer: Cellular and Molecular Biology*. Boston: Kluwer, 1988.
- Dickson, R. B., Huff, K. K., Spencer, E. M., and Lippman, M. E. Induction of epidermal growth factor-related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. *Endocrinology*, 118: 138–142, 1986.
- Bates, S. E., McManaway, M. E., Lippman, M. E., and Dickson, R. B. Characterization of estrogen responsive transforming activity in human breast cancer cell lines. *Cancer Res.*, 46: 1707–1713, 1986.
- Dubik, D., Dembinski, T. C., and Shiu, R. P. C. Stimulation of c-myc oncogene expression associated with estrogen-induced proliferation of human breast cancer cells. *Cancer Res.*, 47: 6517–6521, 1987.
- Dubik, D., and Shiu, R. P. C. Transcriptional regulation of c-myc oncogene expression by estrogen in hormone-responsive human breast cancer cells. *J. Biol. Chem.*, 263: 12705–12708, 1988.
- Musgrove, E. A., Lee, C. S. L., and Sutherland, R. L. Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor α, epidermal growth factor receptor, c-fos, and c-myc genes. *Mol. Cell. Biol.*, 11: 5032–5043, 1991.
- Kenney, N. J., Saeki, T., Gottardis, M., Kim, N., Garcia-Morales, P., Martin, M. B., Normanno, N., Ciardello, F., Day, A., Cutler, M. L., and Salomon, D. S. Expression of transforming growth factor α antisense mRNA inhibits the estrogen-induced production of TGFα and estrogen-induced proliferation of estrogen-responsive human breast cancer cells. *J. Cell. Physiol.*, 156: 497–514, 1993.
- Watson, P. H., Pon, R. T., and Shiu, R. P. C. Inhibition of c-myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer. *Cancer Res.*, 51: 3996–4000, 1991.
- Jhappan, C., Stahle, C., Harkins, R. N., Fausto, N., Smith, G. H., and Merlino, G. T. TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. *Cell*, 61: 1137–1146, 1990.
- Matsui, Y., Halter, S. A., Holt, J. T., Hogan, B. L. M., and Coffey, R. J. Development of mammary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. *Cell*, 61: 1147–1155, 1990.
- Sandgren, E. P., Lutteke, N. C., Palmiter, R. D., Brinster, R. L., and Lee, D. C. Overexpression of TGFα in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. *Cell*, 61: 1121–1135, 1990.
- Stewart, T. A., Pattengale, P. K., and Leder, P. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. *Cell*, 38: 627–637, 1984.
- Land, H., Parada, L. F., and Weinberg, R. A. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature (Lond.)*, 304: 596–602, 1983.

26. Lee, W. M. F., Schwab, M., Westaway, D., and Varmus, H. E. Augmented expression of normal *c-myc* is sufficient for cotransformation of rat embryo cells with a mutant *ras* gene. *Mol. Cell. Biol.*, 5: 3345–3356, 1985.
27. Khazaie, K., Panayotou, G., Aguzzi, A., Samarut, J., Gazzolo, L., and Jurdic, P. EGF promotes *in vivo* tumorigenic growth of primary chicken embryo fibroblasts expressing *v-myc* and enhances *in vitro* transformation by the *v-erbA* oncogene. *Oncogene*, 6: 21–28, 1991.
28. Stern, D. F., Roberts, A. B., Roche, N. S., Sporn, M. B., and Weinberg, R. A. Differential responsiveness of *myc*- and *ras*-transfected cells to growth factors: selective stimulation of *myc*-transfected cells by epidermal growth factor. *Mol. Cell. Biol.*, 6: 870–877, 1986.
29. Telang, N. T., Osborne, M. P., Sweterlitsch, L. A., and Narayanan, R. Neoplastic transformation of mouse mammary epithelial cells by deregulated *myc* expression. *Cell Regul.*, 1: 863–872, 1990.
30. Valverius, E. M., Ciardello, F., Heldin, N. E., Blondel, B., Merlo, G., Smith, G., Stampfer, M. R., Lippman, M. E., Dickson, R. B., and Salomon, D. E. Stromal influences on transformation of human mammary epithelial cells overexpressing *c-myc* and SV40T. *J. Cell. Physiol.*, 145: 207–216, 1990.
31. Murakami, H., Sanderson, N. D., Nagy, P., Marino, P. A., Merlino, G., and Thorgerisson, S. S. Transgenic mouse model for synergistic effects of nuclear oncogenes and growth factors in tumorigenesis: interaction of *c-myc* and transforming growth factor  $\alpha$  in hepatic oncogenesis. *Cancer Res.*, 53: 1719–1723, 1993.
32. Sandgren, E. P., Luetkeke, N. C., Qiu, T. H., Palmiter, R. D., Brinster, R. L., and Lee, D. C. Transforming growth factor  $\alpha$  dramatically enhances oncogene-induced carcinogenesis in transgenic mouse pancreas and liver. *Mol. Cell. Biol.*, 13: 320–330, 1993.
33. Luetkeke, N. C., Lee, D. C., Palmiter, R. D., Brinster, R. L., and Sandgren, E. P. Regulation of fat and muscle development by transforming growth factor  $\alpha$  in transgenic mice and in cultured cells. *Cell Growth & Differ.*, 4: 203–213, 1993.
34. Takagi, H., Jhappan, C., Sharp, R., and Merlino, G. Hypertrophic gastropathy resembling Meinkrantz's disease in transgenic mice overexpressing transforming growth factor  $\alpha$  in the stomach. *J. Clin. Invest.*, 90: 1161–1167, 1992.
35. Cardiff, R. D., Sinn, E., Muller, W., and Leder, P. Transgenic oncogenic mice: tumor phenotype predicts genotype. *Am. J. Pathol.*, 139: 495–501, 1991.
36. Guy, C., Cardiff, R. D., and Muller, W. J. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol. Cell. Biol.*, 12: 954–961, 1992.
37. Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., and Leder, P. Single step induction of mammary adenocarcinoma in transgenic mice bearing the activated *c-neu* oncogene. *Cell*, 54: 105–115, 1988.
38. Bouchard, L., Lamarre, L., Tremblay, P. J., and Jolicoeur, P. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/*c-neu* oncogene. *Cell*, 57: 931–936, 1989.
39. Lucchini, F., Sacco, M. G., Hu, N., Villa, A., Brown, J., Cesano, L., Mangiarini, L., Rindi, G., Kindl, S., Sessa, F., Vezzoni, P., and Clerici, L. Early and multifocal tumors in breast, salivary, Harderian and epididymal tissues developed in MMTV-*Neu* transgenic mice. *Cancer Lett.*, 64: 203–209, 1992.
40. Cook, D. N., and Hassell, J. A. The amino terminus of polyoma middle T antigen is required for transformation. *J. Virol.*, 64: 1879–1887, 1990.
41. Pallas, D. C., Shahrik, L. K., Martin, B. L., Jaspers, S. L., Miller, T. B., Brautigan, D. L., and Roberts, T. M. Polyoma small and middle T antigens and SV40 small antigens form stable complexes with protein phosphatase 2A. *Cell*, 60: 167–172, 1990.
42. Talmage, D. A., Freund, R., Young, A. T., Dahl, J., Dawe, C. J., and Benjamin, T. L. Phosphorylation of middle T by pp60<sup>c-src</sup>: a switch for binding of phosphatidylinositol 3-kinase and optimal tumorigenesis. *Cell*, 59: 55–65, 1989.
43. Walter, G., Ruediger, R., Slaughter, C., and Mumby, M. Association of protein phosphatase A with polyoma virus medium tumor antigen. *Proc. Natl. Acad. Sci. USA*, 78: 2521–2525, 1990.
44. Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G. H., Merlino, G. T., and Callahan, R. Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Dev.*, 6: 345–355, 1992.
45. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T., and Varmus, H. E. Expression of the *int-1* gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinoma in male and female mice. *Cell*, 55: 619–625, 1988.
46. Jhappan, C., Takayama, H., Dickson, R. B., Merlino, G. Transgenic mice provide genetic evidence that transforming growth factor  $\alpha$  promotes skin tumorigenesis via H-Ras-dependent and H-Ras-independent pathways. *Cell Growth & Differ.*, 5: 385–394, 1994.
47. Clarke, R., Brunner, N., Katz, D., Glanz, P., Dickson, R. B., Lippman, M. E., and Kern, F. G. The effects of a constitutive expression of transforming growth factor- $\alpha$  on the growth of MCF-7 human breast cancer cells *in vitro* and *in vivo*. *Mol. Endocrinol.*, 3: 372–380, 1989.
48. Dean, M., Cleveland, J. L., Rapp, U., and Ihle, J. N. Role of *myc* in the abrogation of IL3 dependence of myeloid FDC-P1 cells. *Oncogene Res.*, 1: 279–296, 1987.
49. Kurebayashi, J., McLeskey, S. W., Johnson, M. D., Lippman, M. E., Dickson, R. B., and Kern, F. G. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells co-transfected with fibroblast growth factor-4 and LacZ. *Cancer Res.*, 53: 2178–2187, 1993.
50. Takagi, H., Sharp, R., Takayama, H., Anver, M. R., Ward, J. M., and Merlino, G. Collaboration between growth factors and diverse chemical carcinogens in hepatocarcinogenesis of transforming growth factor  $\alpha$  transgenic mice. *Cancer Res.*, 53: 4329–4336, 1993.
51. Hung, M. C., Thompson, K. L., Chiu, I. M., and Rosner, M. R. Characterization of rodent epidermal growth factor receptor transcripts using a mouse genomic probe. *Biochem. Biophys. Res. Commun.*, 141: 1109–1115, 1986.
52. Fox, C., and Cottler, T. *In situ* hybridization for the detection of HIV RNA in cells and tissues. In: J. Coligan *et al.* (eds.), *Current Protocols in Immunology*. New York: John Wiley and Sons, 1993.
53. Tamano, S., Merlino, G., and Ward, J. M. Rapid development of hepatic tumors in transforming growth factor  $\alpha$  transgenic mice associated with increased cell proliferation in precancerous hepatocellular lesions initiated by N-nitrosodiethylamine. *Carcinogenesis (London)*, 15: 1791–1798, 1994.